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Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*

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Supporting Information

Production of the mammalian hormone melatonin from glucose in *Saccharomyces cerevisiae*.

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Abbreviations

Enzymes and their corresponding EC numbers.

Abbreviation	Enzyme	EC #
AANAT	serotonin acetyltransferase	EC 2.3.1.87 or EC 2.3.1.5
ACS	acetyl-CoA synthase	EC 2.3.1.169
ALD6	aldehyde dehydrogenase	EC 1.2.1.4
ARO9	aromatic aminotransferase II	EC 2.6.1.57
ASMT	acetylserotonin O-methyltransferase	EC 2.1.1.4
DDC	5-hydroxy-L-tryptophan decarboxylase, a.k.a. dopa decarboxylase (aromatic L-amino acid decarboxylase)	EC 4.1.1.28
DHPR	dihydropteridine reductase	EC 1.5.1.34
ERC1	ethionine resistance conferring gene	N/A
GCH1	GTP cyclohydrolase I	EC 3.5.4.16
MS	methionine synthase	EC 2.1.1..
PCBD	pterin-4-alpha-carbinolamine dehydratase, a.k.a. 4a-hydroxytetrahydrobiopterin dehydratase	EC 4.2.1.96
PTS a.k.a. PTPS	6-pyruvoyl-tetrahydropterin synthase	EC 4.2.3.12
SAH1	S-adenosyl-homocysteine hydrosylase	EC 3.3.1.1
SAM2	S-adenosylmethionine synthetase	EC 2.5.1.6
SAMS	S-adenosyl-methionine synthetase	EC 2.5.1.6
SPR	sepiapterin reductase	EC 1.1.1.153
TPH	L-tryptophan hydroxylase	EC 1.14.16.4

Metabolites and their corresponding PubChem numbers.

Abbreviation	Metabolite	PubChem CID
Acetyl-CoA	acetyl coenzyme A	444493
BH4/THB	tetrahydrobiopterin	1125
DHB	dihydrobiopterin	119055
DHP	7,8-dihydronoopterin 3'-triphosphate	121885
GTP	guanosine triphosphate	6830
HTHB	4a-hydroxytetra-hydrobiopterin	129803
5-HTP	5-hydroxy-L-tryptophan	144
NADH	nicotinamide adenine dinucleotide	5893

NADPH	nicotinamide adenine dinucleotide phosphate	5884
6-PTH	6-pyruvoyltetrahydropterin	128973
SAM	<i>S</i> -adenosyl- <i>L</i> -methionine	34755
SAH	<i>S</i> -adenosyl- <i>L</i> -homocysteine	439155

Supplementary Methods

Synthetic genes for yeast expression constructs

Genes encoding a *B. taurus* arylalkylamine N-acetyltransferase BtAANAT (*GenBank*: [281583](#)), *H. sapiens* acetylserotonin O-methyltransferase HsASMT (*GenBank*: [438](#)), *H. sapiens* 5-hydroxy-L-tryptophan decarboxylyase HsDDC (*GenBank*: [1644](#)), *H. sapiens* 6-pyruvoyl-tetrahydropterin synthase HsDHPR (*GenBank*: [5860](#)), *R. norvegicus* 6-pyruvoyl-tetrahydropterin synthase RnDHPR (*GenBank*: [64192](#)), *L. ruminis* pterin-4-alpha-carbinolamine dehydratase LrPCBD1 (*GenBank*: [WP_003692157.1](#)), *P. aeruginosa* pterin-4-alpha-carbinolamine dehydratase PaPCBD1 (*GenBank*: [880827](#)), *R. norvegicus* 6-pyruvoyl-tetrahydropterin synthase RnPTPS a.k.a. RnPTS (*GenBank*: [29498](#)), *R. norvegicus* sepiapterin reductase RnSPR (*GenBank*: [29270](#)), a double truncated *H. sapiens* tryptophan hydroxylase HsTPH2₁₄₆₋₄₆₀ (Q8IWU9) (*GenBank*: [121278](#)), and *S. mansoni* tryptophan hydroxylase SmTPH (*GenBank*: [AF031034.1](#)) were synthesized by GeneArt (Life Technologies) in versions codon-optimized for yeast *S. cerevisiae* or *E. coli* (as indicated in Table s2). The synthetic gene constructs had a general structure: **ATGNN...NNTGA**, where ATG is the start codon, NN...NN represents the protein coding sequence without start and stop codons, TGA (or alternatively TAA) is the stop codon. The Kozak sequence (AAAACA) upstream of the start codon for improved yeast transcription was introduced via the gene BioBrick primers (Table s1).

Cloning of yeast expression constructs

The gene fragments (BioBricks) carrying the genes and correct overhangs for USER-cloning were generated by PCR amplification using primers and templates as indicated in Table s2. The PCR mix contained: The PCR mix contained: 18 µl water, 10 µl HF phusion buffer (5x, BioLab), 5 µl 2mM dNTP, 2 µl PfuX7 polymerase [1], 2.5 µl forward primer (10 µM), 2.5 µl reverse primer (10 µM), and 1 µl DNA template. The cycling program was: 95°C for 2 min, 30 cycles of [95°C for 10 sec, 52°C for 20 sec, 68°C for (1min/kb)], 68°C for 5 min, pause at 10°C. The gene fragments were resolved on 1% agarose gel containing SYBR®-SAFE (Invitrogen) and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The promoter fragments were also generated by PCR followed by DNA purification (Table s2). The terminators were already present on the yeast vectors. The expression plasmids were created by USER-cloning as described previously using *E. coli* strain DH5alpha [2]. The clones with correct inserts were identified by colony PCR and the plasmids of 4 clones/transformation were

isolated from overnight *E. coli* cultures and confirmed by sequencing (Eurofins). The expression plasmids are listed in Table s3, and the primers used are listed in Table s1. Construction of plasmids pCfB2772 and pCfB2773 was mainly performed on the Hamilton Vantage Cloning Robot.

In the course of this study, plasmid pCfB998 was found to have acquired the point mutation G163C in BtAANAT. Since this caused the altered translated protein BtAANAT-A55P, we fixed this mutation by site-directed mutagenesis to generate pCfB2628 using primers PR-8518/PR-8519 and the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions.

For subcloning HsASMT into a high-copy 2 μ vector, we created a terminator-gene1-promoter1 BioBrick with primers PR-6/PR-11055 on template pCfB1252, and USER cloned this BioBrick into USER-prepared pESC-LEU-USER cloning site (SEQ ID NO: 15).

Construction of yeast production strains

The yeast expression plasmids were transformed into *S. cerevisiae* cells using the lithium acetate transformation protocol [3]. Prior to transformation, integrative vectors were digested by *NotI* and column-purified (Nucelospin Gel and PCR cleanup kit, Macherey Nagel). Approximately 1 μ g DNA (single integrative) or 1.5 μ g (multi-copy integrative, Ty2) was transformed into competent yeast cells. The cells were selected on drop-out agar medium, and correct integration at the specific genomic loci was verified by colony PCR. Cre-loxP-mediated selection marker loop out was performed as described previously [2]. After loop out, colony PCR was performed to confirm the retained presence of the integrated markerless genes using the same primer sets for cloning the initial gene BioBricks (Table s1). The resulting yeast strains are listed in Table s4.

The *aro9 Δ* deletion strain was constructed by amplifying the KanMX-deleted ORF from genomic DNA of the *aro9 Δ ::KanMX* deletion strain of the YKO MATA Strain Collection (open biosystems), using primers PR-13241/PR-13242, and transforming 1 μ g of this deletion cassette into yeast. Correct integration was confirmed by colony PCR with primers PR-477/PR-13441.

The plasmids and strains described in this work will be readily provided on request to the corresponding author.

Metabolite analysis by LC-ESI-MS

LC-ESI-MS data was collected on OrbiTrap Fusion High Resolution Mass Spectrometer system coupled with an Ultimate 3000 UHPLC pump (Thermo, San Jose Ca). Samples were held in the autosampler at a temperature of 10.0°C during the analysis. 1 μ L Injections of the sample were made onto a Thermo HyperSil Gold PFP HPLC column, with a 3 um particle size, 2.1 mm i.d. and 150 mm long. The column was held at a temperature of 35.0°C. The solvent system used was Solvent A "Water with 0.1% formic acid" and Solvent B "Acetonitrile with 0.1% formic ". The Flow Rate was 1.000 ml/min with an Initial Solvent composition of %A = 95, %B = 5 held until 0.50 min, the solvent composition was then changed following a Linear Gradient until it reached %A = 70.0 and %B = 30.0 at 1.50 min. The solvent composition was then changed following a Linear Gradient until it reached %A = 5.0 and %B = 95.0 at 2.00 min This was held until 2.50 min when the solvent was returned to the initial conditions and the column was re-equilibrated until 3.00 min. The first 0.25 min of the run was diverted to waste using the divert valve, following which the column eluent flowed directly into the Heated ESI probe of the MS which was held at 325°C and a voltage of 3500 V. Data was collected in positive ion mode over the mass range 50 to 1000 m/z at a resolution of 15.000. The other MS settings were as follows, Sheath Gas Flow Rate of 60 units, Cone Gas Flow Rate of 20 units Cone Temp was 275°C.

Cell harvest, DNA purification and total RNA extraction, and reverse transcription

Strains were grown overnight at 30°C in SC-ura media. The next day, 2.4 x 10⁸ cells were harvested for DNA extraction, and kept at -20°C until further processing. For RNA extraction, the remaining cultures were diluted to OD₆₀₀ = 0.2 and grown to mid-log phase (OD₆₀₀ ~ 0.5). Then 3.6 x 10⁸ cells were harvested, snap frozen in liquid nitrogen, and kept at -80°C until further processing. Purified genomic DNA was prepared as described before [4]. Total RNA extraction was performed using the PureLink® RNA Mini Kit (Ambion, Life Technologies). Cells were lysed enzymatically for 1h at 30°C with Zymolase® 100T (amsbio) according to manufacturers' instructions. RNA samples were then treated with DNaseI for 30 min at 37°C, and DNaseI subsequently inactivated. Isolated total RNA integrity was verified by agarose gel electrophoresis and SYBR® Safe DNA gel staining (Thermo Fisher Scientific) of samples denatured by formamide (Sigma), and by an average UV absorbance ratio A₂₆₀:A₂₈₀ of 2.14 (range 2.1 – 2.2). 500 ng total RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Thermo Scientific) using random hexamer primers according to manufacturers' instructions, and included a non-template control (NTC), the manufacturer's positive control (PC), and

control reactions not containing reverse transcriptase (-RT) to verify the absence of genomic DNA.

Quantitative real-time PCR (QPCR) and reverse-transcription quantitative real-time PCR (RT-QPCR)

The copy numbers of the HsTPH2₁₄₆₋₄₆₀ and the SmTPH genes were determined by quantitative real-time PCR (QPCR) analysis, and mRNA levels by reverse-transcription quantitative real-time PCR (RT-QPCR) using primer pair PR-14437/PR-14438 or PR-13245/PR-13256, respectively. All values were normalized to the reference gene *ACT1*, amplified with primers PR-14439/PR-14440. All QPCR and RT-QPCR reactions were performed in the real-time thermal cycler Mx3005P (Stratagene, Agilent Technologies) using the SYBR® Select Master Mix (Life Technologies) with 0.08μl DNA or 2μl cDNA as template. For standard curves, DNA/cDNA of a strain with a single copy of the respective TPH was used (HsTPH2₁₄₆₋₄₆₀ : SCE-iL3-HM-48_1; SmTPH: SCE-iL3-HM-49_1). PCR plates were sealed with MicroAmp® Optical Adhesive Film (Life Technologies). The following PCR cycling conditions were used: (i) UDG activation (2 min at 50°C); (ii) AmpliTaq® DNA polymerase, UP activation (2 min at 95°C); (iii) amplification and quantification program repeated 40 times (15 sec at 95°C; 30 sec at 54°C; 1 min at 72°C with a single fluorescence measurement); (iv) melting curve program (1 min at 95°C; 54-95°C with a continuous fluorescence measurement); (v) cooling program down to 12°C. Calibration curves, efficiencies, correlation coefficients, and threshold cycles (C_T) for the amplification curves are available on request. Data was analyzed using the MxPro QPCR Software for Mx3000P and Mx3005P QPCR Systems (Stratagene, Agilent Technologies) and Microsoft Excel. Specificity was confirmed empirically by gel electrophoresis and melting curve profiles. Non-template controls (NTCs) were performed to detect PCR contaminations and primer dimers. All reactions were measured in triplicates.

Supplementary Figures

Figure s1. Production pathway for *de novo* biosynthesis of melatonin in *S. cerevisiae* and its connection to the intrinsic yeast metabolic pathways. Central yeast metabolic pathways, substrate synthesis, energy metabolism, transporters and the interface to the heterologous pathways are shown.

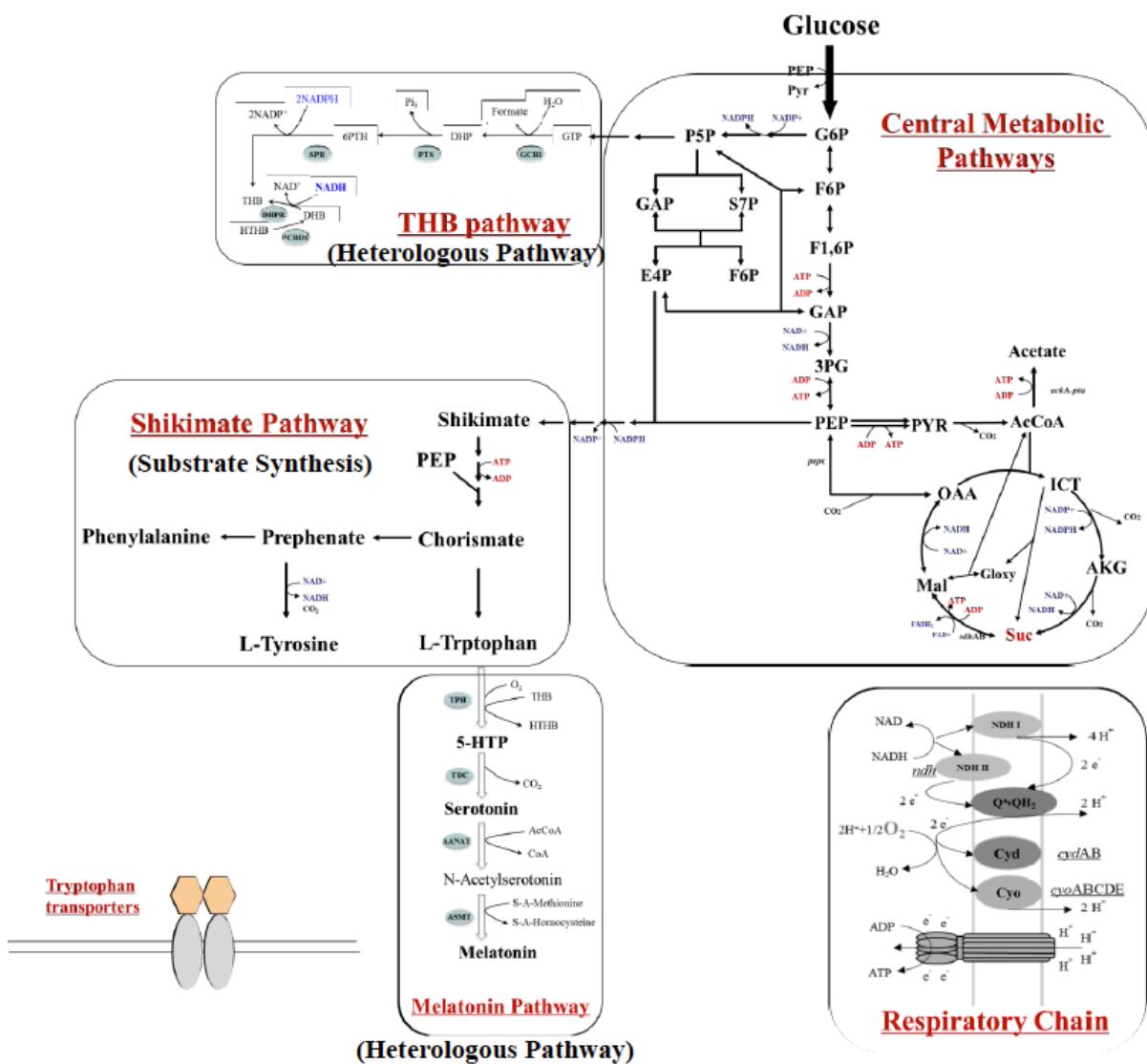


Figure s2. Clonal reproducibility of metabolite production in yeast strains expressing the melatonin biosynthesis pathway from glucose. (A) HPLC analysis of bioconversion products of individual clones of *S. cerevisiae* strain SCE-iL3-HM-43 (SmTPH PaPCBD1 RnDHPR RnPTS RnSPR HsDDC BtAANAT HsASMT). (i) melatonin standard (control), (ii) clone 7, (iii) clone 12, (iv) clone 13, (v) clone 17, (vi) clone 22, and (vii) clone 40. Compound 3 (melatonin) has a retention time of 2.2 min. (B) LC/ESI-MS analysis of metabolites from the *S. cerevisiae* clones described in panel A in the positive mode: exact mass of compound 3 (melatonin) $[M + H]^+$ [m/z] (233.128).

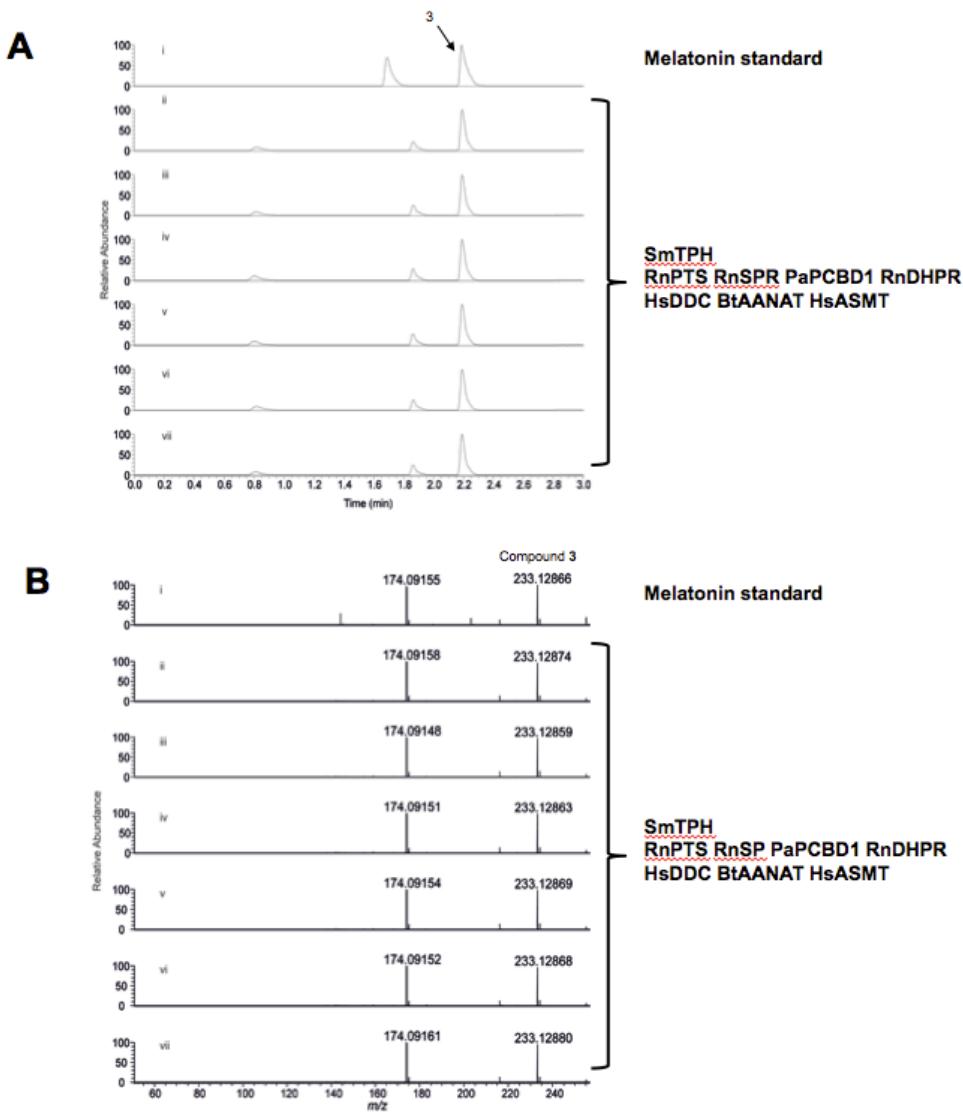
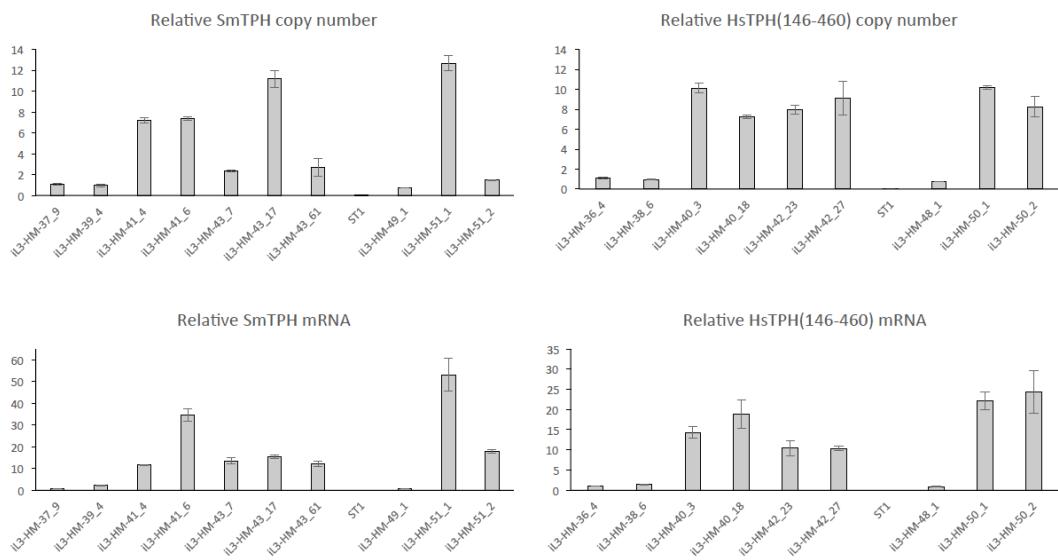


Figure s3. Analysis of SmTPH and HsTPH₁₄₆₋₄₆₀ copy number and mRNA levels in the respective production strains. Strains were cultured in SC-ura and harvested for DNA or RNA extraction, and subsequently QPCR or RT-QPCR was performed with primer pairs for SmTPH, HsTPH₁₄₆₋₄₆₀, and ScACT1 as reference. Relative SmTPH and HsTPH₁₄₆₋₄₆₀ copy number and mRNA levels were found by normalizing to ScACT1. All reactions were performed in triplicate, error bars represent SD. Numerical values are shown in table s6.



Supplementary Tables

Table s1: Primer sequences. Sequences of primers (5' to 3') used in this study. Overhangs used for USER cloning are underlined.

Primer ID	Primer name	Primer sequence, 5' to 3'
PR-5	PTEF1-fw	<u>ACCTGCACU TTGTAATTAAAACCTAG</u>
PR-6	PTEF1-rv	<u>CACCGCAU GCACACACCATACTTC</u>
PR-7	PPGK1-fw	<u>CGTGCAGAU GGAAGTACCTTCAAAGA</u>
PR-8	PPGK1-rv	<u>ATGACAGAU TTGTTTTATATTGTTG</u>
PR-278	pTDH3_fw	<u>CGTGCAGAU ATAAAAAACACGCTTTTCAGTCG</u>
PR-279	pTDH3_rv	<u>ATGACAGAU TTTGTTTGTATGTGTGTTATT</u>
PR-394	RnSPR-fw	<u>ATCTGTCAU AAAACAATGAAAGGAGGCAGGCTAG</u>
PR-389	RnSPR-rv	<u>CACCGCAU TTAAATGTCATAGAAGTCCACGTG</u>
PR-393	RnPTS-fw	<u>AGTGCAGGU AAAACAATGAACGCCGGTTGG</u>
PR-350	RnPTS-rv	<u>CGTGCAGAU TTATTCTCCTTTGAGACCAAT</u>
PR-477	KanMX_2/3_START_rv	AGTGACGACTGAATCCGGTG
PR-738	ALD6_U2_fw	<u>ATCTGTCAU AAAACAATGACTAAAGCTACACTTGACAC</u>
PR-739	ALD6_U2_rv	<u>CACCGCAU TCACAACCTAATTCTGACAGCTTTAC</u>
PR-1759	HsDDC-rv	<u>CGTGCAGAU TTATTTCACGTTGCCACGCAC</u>
PR-1760	HsDDC-fw	<u>AGTGCAGGU AAAACAATGAATGCAAGCGAATTCTCG</u>
PR-1761	BtAANAT-fw	ATCTGTCAU AAAACAATGAGCACCCGAGCATTCTTG
PR-1762	BtAANAT-rv	<u>CACCGCAU TTAACGATCGCTATTACGACGCAGTG</u>
PR-1764	GgASMT-fw	<u>AGTGCAGGU AAAACAATGGATAGCACCGAAGATCTGG</u>
PR-1763	GgASMT-rv	<u>CGTGCAGAU TTATTTACGACCCAGAACTGCATC</u>
PR-2148	PaPCBD1-fw	<u>AGTGCAGGU AAAACAATGACTGCTTTGACTCAAGC</u>
PR-2149	PaPCBD1-rv	<u>CGTGCAGAU TTACTTTCTACCTTCAGCAG</u>
PR-2150	LrPCBD1-fw	<u>AGTGCAGGU AAAACAATGGTCAAGTTGTTCCCAC</u>
PR-2151	LrPCBD1-rv	<u>CGTGCAGAU TCAAATTCTGGCATCTGAATTTC</u>
PR-2152	HsDHPR-fw	<u>ATCTGTCAU AAAACAATGGCTGCTGCTG</u>
PR-2153	HsDHPR-rv	<u>CACCGCAU TTAGAAGTAAGCTGGAGTC</u>
PR-2154	RatDHPYeast-fw	<u>ATCTGTCAU AAAACAATGGCTTCTGG</u>
PR-2155	RatDHPYeast-rv	<u>CACCGCAU TTAGAAGTAAGCTGGAGTCATT</u>
PR-2254	HsASMT-fw	<u>AGTGCAGGU AAAACAATGGTAGCAGCGAAGATC</u>
PR-2255	HsASMT-rv	<u>CGTGCAGAU TTATTACGTGCCAGGATTGCATC</u>
PR-8502	SmTPH-fw	<u>ATCTGTCAU AAAACAATGATTAGCACCGAAAGCG</u>
PR-8503	SmTPH-rv	<u>CACCGCAU TTAGCTGCTGCGATTTCG</u>
PR-8504	HsTPH2-(146-460)-fw	<u>ATCTGTCAU AAAACAATGGAACCTGGAAAGATGTTCCG</u>
PR-8505	HsTPH2-(146-460)-rv	<u>CACCGCAU TTAGGTATTTCAGGATCTCGAT</u>
PR-8518	BtAANAT correction-fw	GTTTTGAAATTGAAACGTGAAGCATTATTAGCGTGAGCGG
PR-8519	BtAANAT correction-rv	CCGCTCACGCTAAATAATGCTTACGTTCAATTCAAAAC
PR-11055	TADH1_U1_fw	<u>CGTGCAGAU GAGCGACCTCATGCTATACC</u>
PR-13235	SeACS_U2_F	<u>ATCTGTCAU AAAACAATGTCACAAACACAC</u>

Primer ID	Primer name	Primer sequence, 5' to 3'
PR-13236	SeACS_U2_R	<u>CACCGGAU</u> TCATGATGGCATAGCAATAG
PR-13237	ScSAM2_U2_F	<u>ATCTGTCAU</u> AAAACAATGTCCAAGAGCAAAACTTCTTATTAC
PR-13238	ScSAM2_U2_R	<u>CACCGGAU</u> TTAAAATTCCAATTCTTTGGTTTTCCC
PR-13239	ScERC1_U2_F	<u>ATCTGTCAU</u> AAAACAATGTCTAACAACTTAGTCATACCACCAAC
PR-13240	ScERC1_U2_R	<u>CACCGGAU</u> CTAGTTATACCCAACCATAAGCCG
PR-13241	ARO9_up_F	GGTAGATAAGAGAGCGGAGCACGTGG
PR-13242	ARO9_down_R	GGAGAGAACAAATGGATAAGTTGCCTTCCTC
PR-13441	ARO9_dw_seq_R	GGAAAACGCAAGTGGATAAAGGGGTGGG
PR-13245	SmTPH_int_F	GAGCCATCATCAGAACGTTCAAGAAATG
PR-13246	SmTPH_int_R	CAATACACGGAACCTCAACATGAACCAGC
PR-14437	HsTPH_int_F2	CGCTGCTGACCAAATATTGTGG
PR-14438	HsTPH_int_R2	CAGCAGCGGCACATGACC
PR-14439	ACT1_F1	ACTGAAGCTCCAATGAACCCCTA
PR-14440	ACT1_R1	GTCCAAGGCGACGTAACATACT

Table s2: DNA BioBricks for yeast expression plasmids. Genes and promoters (BioBricks) were PCR-amplified from the indicated templates using the corresponding forward and reverse oligos.

BioBrick name ^{a)}	Description	Oligo forward	Oligo reverse	Template
<-ScP _{TEF1}	Promoter of <i>TEF1</i> gene (<i>S. cerevisiae</i>)	PR-5	PR-6	pSP-GM1
->ScP _{PGK1}	Promoter of <i>PGK1</i> gene (<i>S. cerevisiae</i>)	PR-7	PR-8	pSP-GM1
<-ScP _{TEF1} - ScP _{PGK1} ->	Fused promoters of <i>TEF1</i> and <i>PGK1</i> genes (<i>S. cerevisiae</i>)	PR-5	PR-8	pSP-GM1
ScP _{TDH3} ->	Promoter of <i>TDH3</i> gene (<i>S. cerevisiae</i>)	PR-278	PR-279	Genomic DNA of CEN.PK113-7D
BtAANAT->	arylalkylamine N-acetyltransferase gene (<i>B. taurus</i>) ^{b)}	PR-1761	PR-1762	pCfB554, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 1
HsASMT<-	acetylserotonin O-methyltransferase gene (<i>H. sapiens</i>) ^{b)}	PR-2254	PR-2255	pCfB560, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 2
HsDDC<-	5-hydroxy-L-tryptophan decarboxylase gene (<i>H. sapiens</i>) ^{b)}	PR-1759	PR-1760	pCfB564, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 3
HsDHPR->	6-pyruvoyl-tetrahydropterin synthase gene (<i>H. sapiens</i>) ^{c)}	PR-2152	PR-2153	pCfB3517, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 4
RnDHPR->	6-pyruvoyl-tetrahydropterin synthase gene (<i>R. norvegicus</i>) ^{c)}	PR-2154	PR-2155	pCfB3518, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 5
LrPCBD1<-	4a-hydroxytetrahydrobiopterin dehydratase 1 gene (<i>L. ruminis</i>) ^{c)}	PR-2150	PR-2151	pCfB3519, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 6
PaPCBD1<-	4a-hydroxytetrahydrobiopterin dehydratase gene (<i>P. aeruginosa</i>) ^{c)}	PR-2148	PR-2149	pCfB3520, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 7
RnPTS-<	6-pyruvoyl-tetrahydropterin synthase gene (<i>R. norvegicus</i>) ^{c)}	PR-393	PR-350	pCfB1205, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 8
RnSPR->	sepiapterin reductase gene (<i>R. norvegicus</i>) ^{c)}	PR-394	PR-389	pCfB1205, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 9
HsTPH2 ₁₄₆₋₄₆₀ ->	double truncated tryptophan hydroxylase 2 gene (<i>H. sapiens</i>)	PR-8504	PR-8505	pCfB2563, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 10
SmTPH->	tryptophan hydroxylase gene (<i>S. mansoni</i>)	PR-8502	PR-8503	pCfB2562, vector with cloned synthetic gene (GeneArt), SEQ ID NO: 11
ScALD6->	cytosolic acetaldehyde dehydrogenase gene (<i>S. cerevisiae</i>)	PR-738	PR-739	Genomic DNA of CEN.PK113-7D
SeACS _{L641P} ->	acetyl-CoA synthase gene with point mutation L641P (<i>S. enterica</i>)	PR-13235	PR-13236	pIYC05
ScSAM2->	S-adenosylmethionine synthetase gene (<i>S. cerevisiae</i>)	PR-13237	PR-13238	Genomic DNA of CEN.PK113-7D
ScERC1->	ethionine resistance conferring gene (<i>S. cerevisiae</i>)	PR-13239	PR-13240	Genomic DNA of CEN.PK113-7D
aro9Δ::KanMX	aromatic aminotransferase II gene (<i>S. cerevisiae</i>)	PR-13241	PR-13242	Genomic DNA of <i>aro9Δ::KanMX</i> strain of YKO MATa Strain Collection (open biosystems)

a) “<-“ indicates gene position 1 and “->” indicates gene position 2 as described in [2]

b) codon-optimized for *E. coli*

c) codon-optimized for *S. cerevisiae*

Table s3: List of plasmids used in this study. The plasmids were constructed by assembling parent plasmid and BioBricks. The resulting relevant gene content, integration site or replicon type and selection marker are shown.

Plasmid name	Parent plasmid, BioBricks	Gene content	Integration site/replicon	Selection marker	Reference/ Source
pESC-URA (pCfB22)	-	-	2m	KIURA3	Agilent
pESC-LEU (pCfB24)	-	-	2m	KILEU2	Agilent
pSP-GM1 (pCfB29)	-	P _{TEF1} , P _{PGK1}	2m	URA3	[5]
pCfB255	-	USER cloning site	X-2	loxP-KIURA3	[2]
pCfB257	-	USER cloning site	X-3	loxP- KILEU2	[2]
pCfB258	-	USER cloning site	X-4	loxP-SpHIS5	[2]
pCfB259	-	USER cloning site	XII-1	loxP-KILEU2	[2]
pIYC05	-	P _{TEF1::SeACS_{L641P}} , P _{PGK1::ScALD6}	2m	HIS3	[6]
pCfB390	-	USER cloning site	XI-3	loxP-KIURA3	[2]
pCfB391	-	USER cloning site	XI-5	loxP-SpHIS5	[2]
pCfB997	pCfB259, GgASMT<-, ScP _{TEF1}	P _{TEF1::GgASMT}	XII-1	loxP-KILEU2	This study
pCfB998	pCfB391, HsDDC<-, ScP _{TEF1} - ScP _{PGK1} , BtAANAT-A55P->	P _{TEF1::HsDDC} , P _{PGK1::BtAANAT_{A55P}}	XI-5	loxP-SpHIS5	This study
pCfB1248	pCfB258, PaPCBD1<-, ScP _{TEF1} - ScP _{PGK1} , RnDHPR->	P _{TEF1::PaPCBD1} , P _{PGK1::RnDHPR}	X-4	loxP-SpHIS5	This study
pCfB1249	pCfB258, LrPCBD1<-, ScP _{TEF1} - ScP _{PGK1} , HsDHPR->	P _{TEF1::LrPCBD1} , P _{PGK1::HsDHPR}	X-4	loxP-SpHIS5	This study
pCfB1251	pCfB257, RnPTS<-, ScP _{TEF1} - ScP _{PGK1} , RnSPR->	P _{TEF1::RnPTS} , P _{PGK1::RnSPR}	X-3	loxP- KILEU2	This study
pCfB1252	pCfB259, HsASMT<-, ScP _{TEF1}	P _{TEF1::HsASMT}	XII-1	loxP- KILEU2	This study
pCfB2224	-	USER cloning site	XI-2	loxP-KanMXsyn	This study
pCfB2528	pCfB390, SmTPH->, ScP _{PGK1}	P _{PGK1::SmTPH}	XI-3	loxP-KIURA3	This study
pCfB2529	pCfB390, HsTPH ₂₁₄₆₋₄₆₀ >, ScP _{PGK1}	P _{PGK1::HsTPH₂₁₄₆₋₄₆₀}	XI-3	loxP-KIURA3	This study
pCfB2628	pCfB998, HsDDC<-, ScP _{TEF1} - ScP _{PGK1} , BtAANAT->	P _{TEF1::HsDDC} , P _{PGK1::BtAANAT}	XI-5	loxP-SpHIS5	This study
pCfB2772	pTY2-loxP-URA3-degron, SmTPH->, ScP _{PGK1}	P _{PGK1::SmTPH}	TY2	loxP-KIURA3-degron	This study SEQ ID NO: 12
pCfB2773	pTY2-loxP-URA3-degron, HsTPH ₂₁₄₆₋₄₆₀ >, ScP _{PGK1}	P _{PGK1::HsTPH₂₁₄₆₋₄₆₀}	TY2	loxP-KIURA3-degron	This study SEQ ID NO: 13
pCfB3337	pESC-LEU-USER cloning site, TADH1, HsASMT<-, ScP _{TEF1}	P _{TEF1::HsASMT}	2μ	NatMX	This study SEQ ID NO: 14
pCfB4149	pCfB2224, SeACS->, ScP _{TDH3}	P _{TDH3::SeACS}	XI-2	loxP-KanMXsyn	This study
pCfB4150	pCfB2224, ScALD6->, ScP _{TDH3}	P _{TDH3::ScALD6}	XI-2	loxP-KanMXsyn	This study
pCfB4151	pCfB2224, ScSAM2->, ScP _{TDH3}	P _{TDH3::ScSAM2}	XI-2	loxP-KanMXsyn	This study
pCfB4152	pCfB2224, ScERC1->, ScP _{TDH3}	P _{TDH3::ScERC1}	XI-2	loxP-KanMXsyn	This study

Table s4: List of strains used in this study. The strains were constructed by transforming plasmids into yeast chassis (parent strains).

Strain name	Parent strain (chassis)	Added plasmid	Relevant genotype	Reference/ Source
CEN.PK113-7D	-	-	<i>MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2</i>	Peter Kötter
CEN.PK102-5B	-	-	<i>MATa ura3-52 his3 D 1 leu2-3/112 MAL2-8c SUC2 [ura- his- leu-]</i>	Peter Kötter
ST3725	BY4741	-	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aro9Δ::KanMX [ura- his- leu- met- G418R]</i>	YKO <i>MATa</i> Strain Collection (open biosystems)
ST892	CEN.PK102-5B	pCfB22, pCfB997, pCfB998	$P_{TEF1}::HsDDC P_{PGK1}::BtAANAT-A55P loxP-SpHIS5$ $P_{TEF1}::GgASMT loxP-KILEU2$ $loxP-KIURA3$	This study
SCE-iL3-HM-11	CEN.PK102-5B	pCfB1249, pCfB1251, pCfB2529	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR loxP-SpHIS5$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR loxP-KILEU2$ $P_{PGK1}::HsTPH2146-460 loxP-KIURA3$	This study
SCE-iL3-HM-12	CEN.PK102-5B	pCfB1249, pCfB1251, pCfB2528	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR loxP-SpHIS5$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR loxP-KILEU2$ $P_{PGK1}::SmTPH loxP-KIURA3$	This study
SCE-iL3-HM-13	CEN.PK102-5B	pCfB1248, pCfB1251, pCfB2528	$P_{TEF1}::PaPCBD1 P_{PGK1}::RnDHPR loxP-SpHIS5$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR loxP-KILEU2$ $P_{PGK1}::HsTPH2146-460 loxP-KIURA3$	This study
SCE-iL3-HM-14	CEN.PK102-5B	pCfB1248, pCfB1251, pCfB2528	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR loxP-SpHIS5$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR loxP-KILEU2$ $P_{PGK1}::SmTPH loxP-KIURA3$	This study
SCE-iL3-HM-19	SCE-iL3-HM-11	SpHIS5, KILEU2 and KIURA3 markers removed by CreA-loxP recombination	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460 [ura- his- leu-]$	This study
SCE-iL3-HM-20	SCE-iL3-HM-12	SpHIS5, KILEU2 and KIURA3 markers removed by CreA-loxP recombination	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR$ $P_{PGK1}::SmTPH [ura- his- leu-]$	This study
SCE-iL3-HM-21	SCE-iL3-HM-13	SpHIS5, KILEU2 and KIURA3 markers removed by CreA-loxP recombination	$P_{TEF1}::PaPCBD1 P_{PGK1}::RnDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460 [ura- his- leu-]$	This study
SCE-iL3-HM-22	SCE-iL3-HM-14	SpHIS5, KILEU2 and KIURA3 markers removed by CreA-loxP recombination	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR$ $P_{PGK1}::SmTPH [ura- his- leu-]$	This study
SCE-iL3-HM-23	SCE-iL3-HM-19	pCfB1252, pCfB2628	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460$ $P_{TEF1}::HsDDC P_{PGK1}::BtAANAT loxP-SpHIS5$ $P_{TEF1}::HsASMT loxP-KILEU2 [ura-]$	This study
SCE-iL3-HM-24	SCE-iL3-HM-20	pCfB1252, pCfB2628	$P_{TEF1}::LrPCBD1 P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS P_{PGK1}::RnSPR P_{PGK1}::SmTPH$ $P_{TEF1}::HsDDC P_{PGK1}::BtAANAT loxP-SpHIS5$ $P_{TEF1}::HsASMT loxP-KILEU2 [ura-]$	This study

Table s4 continued

Strain name	Parent strain (chassis)	Added plasmid	Relevant genotype	Reference/ Source
SCE-iL3-HM-25	SCE-iL3-HM-21	pCfB1252, pCfB2628	$P_{TEF1}::PaPCBD1\ P_{PGK1}::RnDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2\ [ura^+]$	This study
SCE-iL3-HM-35	SCE-iL3-HM-22	pCfB1252, pCfB2628	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR\ P_{PGK1}::SmTPH$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2\ [ura^+]$	This study
SCE-iL3-HM-36	SCE-iL3-HM-23	pCfB255	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $loxP-KIURA3$	This study
SCE-iL3-HM-37	SCE-iL3-HM-24	pCfB255	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR\ P_{PGK1}::SmTPH$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $loxP-KIURA3$	This study
SCE-iL3-HM-38	SCE-iL3-HM-25	pCfB255	$P_{TEF1}::PaPCBD1\ P_{PGK1}::RnDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $loxP-KIURA3$	This study
SCE-iL3-HM-39	SCE-iL3-HM-35	pCfB255	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR\ P_{PGK1}::SmTPH$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $loxP-KIURA3$	This study
SCE-iL3-HM-40	SCE-iL3-HM-23	pCfB2773	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR$ $P_{PGK1}::HsTPH2146-460$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $(P_{PGK1}::HsTPH146-460\ loxP-KIURA3-degron)_n\ ^a)$	This study
SCE-iL3-HM-41	SCE-iL3-HM-24	pCfB2772	$P_{TEF1}::LrPCBD1\ P_{PGK1}::HsDHPR$ $P_{TEF1}::RnPTS\ P_{PGK1}::RnSPR\ P_{PGK1}::SmTPH$ $P_{TEF1}::HsDDC\ P_{PGK1}::BtAANAT\ loxP-SpHIS5$ $P_{TEF1}::HsASMT\ loxP-KILEU2$ $(P_{PGK1}::SmTPH\ loxP-KIURA3-degron)_n\ ^a)$	This study

Table s4 continued

Strain name	Parent strain (chassis)	Added plasmid	Relevant genotype*	Reference/ Source
SCE-iL3-HM-42	SCE-iL3-HM-25	pCfB2773	P _{TEF1} ::PaPCBD1 P _{PGK1} ::RnDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::HsTPH2146-460 P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::HsTPH2146-460 loxP-KIURA3-degron) _n a)	This study
SCE-iL3-HM-43	SCE-iL3-HM-35	pCfB2772	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a)	This study
SCE-iL3-HM-44	SCE-iL3-HM-43 clone 7	pCfB3337	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TEF1} ::HsASMT NatMX (2μ) b) [ClonNat ^R]	This study
SCE-iL3-HM-45	SCE-iL3-HM-43 clone 17	pCfB3337	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TEF1} ::HsASMT NatMX (2μ) b) [ClonNat ^R]	This study
SCE-iL3-HM-47	CEN.PK 102-5B	pCfB2628, pCfB1252	P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2	This study
SCE-iL3-HM-48	SCE-iL3-HM-47	pCfB2529	P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 P _{PGK1} ::HsTPH2146-460 loxP-KIURA3	This study
SCE-iL3-HM-49	SCE-iL3-HM-47	pCfB2528	P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 P _{PGK1} ::SmTPH loxP-KIURA3	This study
SCE-iL3-HM-50	SCE-iL3-HM-47	pCfB2773	P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::HsTPH2146-460 loxP-KIURA3-degron) _n a)	This study
SCE-iL3-HM-51	SCE-iL3-HM-47	pCfB2772	P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a)	This study
SCE-iL3-HM-54	SCE-iL3-HM-43 clone 61	aro9Δ::KanMX deletion cassette	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) aro9Δ::KanMX [G418 ^R]	This study

Table s4 continued

Strain name	Parent strain (chassis)	Added plasmid	Relevant genotype*	Reference/ Source
SCE-iL3-HM-57	SCE-iL3-HM-43 clone 61	pCfB4149	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TDH3} ::SeACS loxP-KanMX [G418 ^R]	This study
SCE-iL3-HM-60	SCE-iL3-HM-43 clone 61	pCfB4150	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TDH3} ::ScALD6 loxP-KanMX [G418 ^R]	This study
SCE-iL3-HM-63	SCE-iL3-HM-43 clone 61	pCfB4151	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TDH3} ::ScSAM2 loxP-KanMX [G418 ^R]	This study
SCE-iL3-HM-66	SCE-iL3-HM-43 clone 61	pCfB4152	P _{TEF1} ::LrPCBD1 P _{PGK1} ::HsDHPR P _{TEF1} ::RnPTS P _{PGK1} ::RnSPR P _{PGK1} ::SmTPH P _{TEF1} ::HsDDC P _{PGK1} ::BtAANAT loxP-SpHIS5 P _{TEF1} ::HsASMT loxP-KILEU2 (P _{PGK1} ::SmTPH loxP-KIURA3-degron) _n a) P _{TDH3} ::ScERC1 loxP-KanMX [G418 ^R]	This study

a) (DNA fragment)_n denotes multi-copy integration of the DNA fragment into TY2 regions of the genome.

b) (2μ) refers to an episomal high-copy plasmid with 2μ origin.

Table s5: Production titers of melatonin cell factories. Strains carry genes for overexpressing RnPTS, RnSPR, HsDDC, BtAANAT, HsASMT, and two variants of TPH, PCBD1, and DHPR, respectively. Cells were cultured in mineral medium, the supernatant extracted with ethanol, and metabolites measured by LC-ESI-MS. Standard deviations were calculated based on three individual clones (CEN.PK113-7D, SCE-iL3-HM-36 to -42) or six individual clones (SCE-iL3-HM-43).

Melatonin production from glucose - Single TPH intergration

Strain ID	5-HTP (mg L ⁻¹)	serotonin (mg L ⁻¹)	N-acetylserotonin (mg L ⁻¹)	melatonin (mg L ⁻¹)	BH4 recycling genes	TPH genes
CEN.PK113-7D	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
SCE-iL3-HM-36	0.00 ± 0.00	0.11 ± 0.19	3.99 ± 0.70	0.41 ± 0.16	LrPCBD1/HsDHPR	HsTPH
SCE-iL3-HM-38	0.00 ± 0.00	2.43 ± 4.21	7.47 ± 0.91	0.76 ± 0.20	PaPCBD1/RnDHPR	HsTPH
SCE-iL3-HM-37	0.00 ± 0.00	0.00 ± 0.00	4.09 ± 0.83	0.27 ± 0.16	LrPCBD1/HsDHPR	SmTPH
SCE-iL3-HM-39	0.00 ± 0.00	0.00 ± 0.00	9.14 ± 1.90	0.82 ± 0.19	PaPCBD1/RnDHPR	SmTPH

Melatonin production from glucose - Multiple TPH intergration

Strain ID	5-HTP (mg L ⁻¹)	serotonin (mg L ⁻¹)	N-acetylserotonin (mg L ⁻¹)	melatonin (mg L ⁻¹)	BH4 recycling genes	TPH genes
SCE-iL3-HM-40	0.00 ± 0.00	0.17 ± 0.30	4.88 ± 1.70	0.40 ± 0.07	LrPCBD1/HsDHPR	HsTPH
SCE-iL3-HM-42	0.09 ± 0.02	4.09 ± 0.76	7.24 ± 0.85	0.90 ± 0.21	PaPCBD1/RnDHPR	HsTPH
SCE-iL3-HM-41	0.00 ± 0.00	0.13 ± 0.03	1.47 ± 0.37	0.03 ± 0.00	LrPCBD1/HsDHPR	SmTPH
SCE-iL3-HM-43	0.22 ± 0.08	9.53 ± 3.57	16.69 ± 0.60	1.93 ± 0.19	PaPCBD1/RnDHPR	SmTPH

Table s6: SmTPH and HsTPH₁₄₆₋₄₆₀ copy number and mRNA levels in the respective production strains. Strains were cultured in SC-ura dropout medium and harvested for DNA or RNA extraction, and subsequently QPCR or RT-QPCR was performed with primer pairs for SmTPH, HsTPH₁₄₆₋₄₆₀, and ScACT1 as reference. Relative SmTPH and HsTPH₁₄₆₋₄₆₀ copy number and mRNA levels were found by normalizing to ScACT1. All reactions were performed in triplicate and the standard deviations calculated.

TPH copy number and mRNA expression levels normalized to ACT1

Strain ID	SmTPH copy number	Relative SmTPH mRNA	Clonal variation of mRNA expression
SCE-iL3-HM-37_9	1.07 ± 0.09	0.61 ± 0.14	-
SCE-iL3-HM-39_4	0.98 ± 0.13	2.12 ± 0.09	-
SCE-iL3-HM-41_4	7.19 ± 0.28	11.69 ± 0.20	SCE-iL3-HM-41
SCE-iL3-HM-41_6	7.37 ± 0.18	34.42 ± 2.86	23.06 ± 16.07
SCE-iL3-HM-43_7	2.34 ± 0.09	13.51 ± 1.35	SCE-iL3-HM-43
SCE-iL3-HM-43_17	11.18 ± 0.83	15.35 ± 0.98	13.68 ± 1.59
SCE-iL3-HM-43_61	2.72 ± 0.84	12.18 ± 1.35	
CEN.PK113-7D	0.00 ± 0.00	0.00 ± 0.00	-
SCE-iL3-HM-49_1	0.75 ± 0.03	0.75 ± 0.05	-
SCE-iL3-HM-51_1	12.66 ± 0.72	53.07 ± 7.64	SCE-iL3-HM-51
SCE-iL3-HM-51_2	1.48 ± 0.07	17.94 ± 0.76	35.51 ± 24.85

Strain ID	HsTPH copy number	Relative HsTPH mRNA	Clonal variation of mRNA expression
SCE-iL3-HM-36_4	1.09 ± 0.08	0.98 ± 0.04	-
SCE-iL3-HM-38_6	0.97 ± 0.04	1.40 ± 0.09	-
SCE-iL3-HM-40_3	10.14 ± 0.49	14.32 ± 1.48	SCE-iL3-HM-40
SCE-iL3-HM-40_18	7.27 ± 0.20	18.74 ± 3.51	16.53 ± 3.13
SCE-iL3-HM-42_23	7.96 ± 0.43	10.43 ± 1.86	SCE-iL3-HM-42
SCE-iL3-HM-42_27	9.09 ± 1.69	10.32 ± 0.58	10.38 ± 0.08
CEN.PK113-7D	0.00 ± 0.00	0.00 ± 0.00	-
SCE-iL3-HM-48_1	0.76 ± 0.04	0.91 ± 0.08	-
SCE-iL3-HM-50_1	10.17 ± 0.19	22.13 ± 2.17	SCE-iL3-HM-50
SCE-iL3-HM-50_2	8.27 ± 1.00	24.36 ± 5.29	23.24 ± 1.58

Table s7: Production titers of the best producing melatonin strain in different cultivation media. Analysis of the non-producing strain CEN.PK 113-7D and the best producing strain SCE-iL3-HM-43 that carries genes for overexpressing RnPTS, RnSPR, PaPCBD1, RnDHPR, HsDDC, BtAANAT, HsASMT, and SmTPH(Ty2) under different cultivation conditions. Strains were cultivated in mineral medium or in FIT medium, the total cell suspension extracted with ethanol, and metabolites measured by LC-ESI-MS. Standard deviations were calculated based on two independent clones.

Melatonin production from glucose - Mineral medium (MM) vs FIT medium

Strain ID	media	serotonin (mg/l)	N-acetylserotonin (mg/l)	melatonin (mg/l)	Fold-change in melatonin production*	p-value*
CEN.PK113-7D	MM	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
SCE-iL3-HM-43		65.91 ± 0.99	22.07 ± 0.20	1.42 ± 0.30	-	-
CEN.PK113-7D	FIT	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	NA
SCE-iL3-HM-43		2.02 ± 0.01	43.27 ± 7.05	13.15 ± 2.05	9.23	0.0016, **

* fold-change and p-values were calculated compared to the identical strain in mineral medium.

** p-value highly significant

Table s8: Production titers of melatonin producing strains with additional modifications. All strains are offspring of SCE-iL3-HM-43 clone 61, and carry genes for overexpressing RnPTS, RnSPR, PaPCBD1, RnDHPR, HsDDC, BtAANAT, HsASMT, and SmTPH(Ty2). In addition, either ScARO9 is deleted, or one of the following genes is overexpressed: SeACS, ScALD6, ScSAM2, or ScERC1. Cells were cultured in mineral medium or FIT medium, total cell suspension extracted with ethanol, and metabolites measured by LC-ESI-MS. Standard deviations are based on biological triplicates.

Melatonin production from glucose with additional metabolic engineering - Mineral medium

Strain ID	additional genetic modification	serotonin (mg/l)	N-acetylserotonin (mg/l)	melatonin (mg/l)	Fold-change in melatonin production*	p-value*
CEN.PK113-7D	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
SCE-iL3-HM-43	-	65.91 ± 0.99	22.07 ± 0.20	1.42 ± 0.30	-	-
SCE-iL3-HM-54	<i>aro9Δ</i>	56.56 ± 15.06	22.26 ± 4.21	1.65 ± 0.29	1.16	0.4654, ns
SCE-iL3-HM-57	SeACS	64.32 ± 1.41	22.01 ± 0.09	1.73 ± 0.05	1.21	0.1600, ns
SCE-iL3-HM-60	ScALD6	30.04 ± 0.35	21.78 ± 0.53	1.52 ± 0.07	1.07	0.5992, ns
SCE-iL3-HM-63	ScSAM2	64.71 ± 1.87	20.08 ± 0.81	1.44 ± 0.10	1.01	0.9857, ns
SCE-iL3-HM-66	ScERC1	51.35 ± 11.59	20.68 ± 2.35	1.57 ± 0.17	1.11	0.5362, ns

Melatonin production from glucose with additional metabolic engineering - FIT medium

Strain ID	additional genetic modification	serotonin (mg/l)	N-acetylserotonin (mg/l)	melatonin (mg/l)	Fold-change in melatonin production*	p-value*
CEN.PK113-7D	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
SCE-iL3-HM-43	-	2.02 ± 0.01	43.27 ± 7.05	13.15 ± 2.05	-	-
SCE-iL3-HM-54	<i>aro9Δ</i>	2.64 ± 0.27	43.40 ± 0.66	13.14 ± 0.53	1.00	0.9915, ns
SCE-iL3-HM-57	SeACS	1.90 ± 0.33	43.04 ± 3.30	13.84 ± 2.09	1.05	0.7400, ns
SCE-iL3-HM-60	ScALD6	2.61 ± 0.07	44.77 ± 2.08	14.50 ± 0.57	1.10	0.3291, ns
SCE-iL3-HM-63	ScSAM2	2.56 ± 0.14	42.83 ± 3.43	10.93 ± 0.19	0.83	0.1344, ns
SCE-iL3-HM-66	ScERC1	2.53 ± 0.68	33.28 ± 10.82	10.72 ± 1.90	0.82	0.2664, ns

* fold-change and p-value were calculated compared to the parent strain (SCE-iL3-HM-43) in the respective medium. ns = not significant.

Sequence Listing. All sequences are protected by the filed patent application [7].

SEQ ID NO: 1

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1 ATGAGCACCC CGAGCATTCA TTGTCTGAAA CCGAGTCCGC TGCATCTGCC GAGCGGTATT
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241 AGCCTGGGTT GGTTGTTGA AGGTGCGTCTG GTTGCATTAA TCATTGGTAG CCTGTGGGAT
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481 GAAGATGCAC TGGTCCCGTT TTATCAGCGT TTTGGTTTC ATCCGGCAGG TCCGTGTGCA
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SEQ ID NO: 2

HsASMT<-

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SEQ ID NO: 3

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 1441 TAA

SEQ ID NO: 4

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421 TTGGATGGTA CTCCAGGTAT GATTGGTTAT GGTATGGCTA AAGGTGCTGT TCATCAATTG
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541 TTGCCAGTTA CTTGGATAC TCCAATGAAC AGAAAGTCTA TGCCAGAAC TGATTTCTCT
601 TCTTGGACTC CATGGAAATT CTTGGTTGAA ACTTTCCATG ATTGGATCAC CGGTAAGAAT
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SEQ ID NO: 5

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SEQ ID NO: 10

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SEQ ID NO: 12

pCfB2772

LOCUS	p2772_(pTY2-KLURA3-TAG-PGK1->SmTPH)	7940 bp	DNA	circular
FEATURES	Location/Qualifiers			
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terminator      complement(284..406)

        /label=tURA3; terminator of K. lactis

misc_structure  complement(407..457)

        /label="Degradation tag"

CDS            complement(458..1258)

        /label=KlURA3 (minus STOP codon)

promoter       complement(1259..1757)

        /label=pURA3; promoter of K. lactis

Misc._feature   1758..1811

        /label=loxP

Misc._feature   1951..2137

        /label=TY2 3'

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        /label=pUC ori

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        /label=AmpR

Misc._feature   4972..5200

        /label=TY2 5'

terminator     complement(5227..5421)

        /label=tADH1; terminator of S. cerevisiae

promoter       5440..6423

        /label=pPGK1; promoter of S. cerevisiae

CDS            6439..7932

        /label=SmTPH

ORIGIN

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SEQ ID NO: 13

pCfB2773

LOCUS	p2773_(pTY2-KLURA3-TAG-PGK1->HsTPH-NC(146-460))	7394 bp
DNA	circular	
FEATURES	Location/Qualifiers	
terminator	6..176	
	/label=tCYC1; terminator of S. cerevisiae	

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Misc._feature 224..283
    /label=loxP

terminator      complement(284..406)
    /label=tURA3; terminator of K. lactis

misc_structure complement(407..457)
    /label="Degradation tag"

CDS            complement(458..1258)
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promoter       complement(1259..1757)
    /label=pURA3; promoter of K. lactis

Misc._feature 1758..1811
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Misc._feature 1951..2137
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rep_origin     complement(2924..3567)
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CDS            complement(3670..4528)
    /label=AmpR

Misc._feature 4972..5200
    /label=TY2 5'

terminator     complement(5227..5421)
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promoter       5440..6423
    /label=pPGK1, promoter of S. cerevisiae

CDS            6439..7386
    /label=HsTPH2-NC(146-460)

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121 TTTCTGTACA GACGCGTGTAA CGCATGTAAC ATTATACTGA AAACCTTGCT TGAGAAATCG
181 CGTCAGCTGA AGCTTCGTAC GCTGCAGGTC GACAACCCTT AATGTCGACA ACCCTTAATA
241 TAACCTCGTA TAATGTATGC TATACGAAGT TATTAGGTCT AGAGATCCC ATACAACAGA
301 TCACCGTGTAC TTTTGTAAGA TGAAGTTGAA GTGAGTGTG CACCGTGCCA ATGCAGGTGG
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421 CGAAATGAGA CAAAGAAGAG AACCAATT TACAAGCATG GGGAGCGCTG ATTCTCTTT
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SEQ ID NO: 14

pCfB3337

LOCUS	p3337 (pESC-NatMXsyn-HsASMT<-PTEF1)	6547 bp	DNA	circular
FEATURES	Location/Qualifiers			
misc_feature	join(4841..4845,1..5) /label=USER cloning site			
rep_origin	189..856 /label=pUC ori			
CDS	complement(1007..1864) /label=AmpR			
rep_origin	1998..3153 /label=2 micron ori			
misc_feature	3533..3919 /label=pTEF1; promoter of A. gossypii			
CDS	3921..4493 /label=nat; Nourseothricin acetyltransferase			
terminator	4494..4742 /label=tTEF1; terminator of A. gossypii			

terminator complement(4854..5048)
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 CDS complement(5067..6104)
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6481 CCGAGAAAAT CTGGAAGAGT AAAAAAGGAG TAGAACATT TTGAAGCTAT GGTGTGTGCA
6541 TCGCGTG

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